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Development of a Quantitative Real-Time PCR Assay for Detection of *Mycoplasma genitalium*

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Mycoplasma genitalium **is known to cause nonchlamydial, nongonococcal urethritis in men and to be associated with pelvic inflammatory disease in women. Specific and sensitive PCR methods are needed for diagnosis of this bacterium because it is very difficult to culture from patient samples. To determine the bacterial load in patients' specimens, a quantitative real-time LightCycler PCR was developed. The housekeeping gene *gap* encoding glyceraldehyde-3-phosphate dehydrogenase was chosen as the target gene. The assay could consistently detect five genome copies per reaction. To evaluate the PCR, we tested 246 selected urethral swab samples from men attending a clinic for sexually transmitted diseases. Eighty-two of the samples were found positive for *M. genitalium* by a conventional 16S rRNA gene PCR assay, whereas 164 samples were randomly chosen among those tested negative. Of the positive samples, 78 (95.1%) were found positive, whereas 6 (3.7%) of the negatives were found positive by the LightCycler assay. The patient samples were also tested with a quantitative TaqMan assay, and the bacterial load was compared to the LightCycler results. A good linear correlation between the LightCycler and the TaqMan assays was found with a correlation coefficient of 0.89 and a slope of 0.99. Significantly more *M. genitalium*-positive men had urethritis, discharge, and dysuria than had *M. genitalium*-negative men. The *M. genitalium* DNA load in samples from patients with urethritis was significantly higher than in samples from those without (61 and 2.9 copies/μl, respectively [$P = 0.0005$]). This assay may prove useful in the monitoring of treatment and for optimizing sample preparation methods.**

Mycoplasma genitalium is a human mycoplasma species, and there is accumulating evidence that it causes nonchlamydial nongonococcal urethritis (NCNGU). In a recent review, the prevalence of *M. genitalium* in male patients with NCNGU was extracted from studies published from 1993 to 2002 (10). The average prevalence of *M. genitalium* was 21.7% of 1,233 patients with NCNGU compared to 6% of 1,537 patients without urethritis. *M. genitalium* is also found in the female genital tract, although the diseases it causes in women have been less thoroughly studied. In women, *M. genitalium* has been associated with cervicitis (16, 26), endometritis (5), and tubal factor infertility (4). The concordance of *M. genitalium* infection among sexual partners implies that *M. genitalium* is sexually transmitted (1, 20).

M. genitalium belongs to the class *Mollicutes*—bacteria without a cell wall. It is the smallest known bacterium, having a genome size of 580 kbp, and is closely related to *Mycoplasma pneumoniae*. Both organisms are flask-shaped with a terminal tip structure that is used for attachment to host cells, and they share several antigens impeding diagnostic serology. It is well established that *M. pneumoniae* causes atypical pneumonia, but occasionally it has been isolated from the urogenital tract (9, 18).

Only a few laboratories have been successful in culturing *M. genitalium* from patient samples; the procedure is very time consuming and slow, taking several months before an isolate is obtained (13, 15, 24, 25). Therefore, PCR has been the only tool to detect *M. genitalium* in clinical studies.

We have developed a quantitative real-time LightCycler PCR assay for the detection of *M. genitalium* in urethral specimens from male patients. The PCR was found to be specific, sensitive, and fast and to have minimal risk of contamination. The clinical performance of the PCR was evaluated using 246 urethral swab specimens from men attending a sexually transmitted disease (STD) clinic, and the results were compared to a conventional *M. genitalium* PCR (12) and a recently developed TaqMan real-time PCR (11).

MATERIALS AND METHODS

Culture of *M. genitalium* strains. *M. genitalium* G37 (American Type Culture Collection) and four Danish strains (M2288, M2300, M2321, and M2341; designated M-strains) isolated from the male urethra (13) were used for this study. Evidence that the four Danish strains were different was shown by sequencing of the *mgpB* gene (11, 17). All strains were grown in 100 ml SP-4 medium (23) in 150-cm^2 TPP tissue culture flasks (TPP, Trasadingen, Switzerland) and incubated at 37°C. The mycoplasmas were harvested in the exponential growth phase, which was indicated by a change in the color of the medium from red to orange. This occurred after 48 h for the type strain G37 and after 3 to 4 days for the M-strains. The SP-4 medium was poured off, and the mycoplasmas attached to the bottom were scraped off in phosphate-buffered saline (PBS; 0.01 M sodium phosphate, 0.85% NaCl, pH 7.4) and pelleted by centrifugation at 15,000 × *g* for 45 min. The cells were resuspended in 4 ml PBS and centrifuged in Eppendorf tubes at 20,000 × *g* for 20 min. The supernatant was discarded, and the pellets were stored at −70°C.

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Culture of other organisms. The following microorganisms and human cells were used in the study. *Mycoplasma fermentans* PG18 was grown in BEg medium (8). *Mycoplasma arthritidis* PG6, *Mycoplasma buccale* 20247, *Mycoplasma hominis* PG21, *Mycoplasma orale* CH19299, and *Mycoplasma salivarium* PG20 were grown in BEa medium (3). *Ureaplasma* type strains *Ureaplasma parvum* serovar 3 and *Ureaplasma urealyticum* serovar 8 were grown in SU medium with 0.04% urea (8). The *Ureaplasma* and *Mycoplasma* strains used in this study were originally obtained from the World Health Organization Collaborating Centre for Mycoplasmas, Aarhus University, Aarhus, Denmark. The mycoplasmas were grown in plastic tubes, and 7.0 ml culture was harvested by centrifugation in Eppendorf tubes at 20,000 × *g* for 30 min. The pellet (from a harvest of 1 ml) was washed twice with PBS. *M. pneumoniae* FH was cultured in SP-4 medium and tissue culture flasks. The mycoplasma pellets were stored at −70°C until use. Culture and harvest of *Chlamydia trachomatis* D (ATCC VR-885) elementary bodies and human HEp2 cells were performed as previously described (19, 27).

DNA extraction. DNA from the mycoplasma strains used in this study, including the M-strains of *M. genitalium*, was released in 200 μl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) with proteinase K (Roche Diagnostics) (200 μg/ml). DNA from chlamydia elementary bodies was purified with the DNeasy tissue kit (QIAGEN GmbH, Hilsen, Germany). The genomic DNA from Hep2 cells was purified with a genomic tip system (QIAGEN Blood and Cell Culture DNA kits). DNA purification was performed in accordance with the manufacturer's instructions.

Patient specimens and clinical information. As part of an ongoing study of the clinical and epidemiological aspects of *M. genitalium* infection, male patients attending an STD clinic in Huddinge, Stockholm, Sweden, between August 1997 and November 2001 were enrolled after providing informed consent. Patients attended the clinic for a checkup for STDs (i.e., being tested for urethritis or human immunodeficiency virus infection or receiving treatment for condyloma) or as part of a contact tracing program of patients with STDs. The local ethical committee approved the study.

Urethral smears were obtained with a plastic loop and stained with methylene blue. Urethritis was defined as ≥5 polymorphonuclear leukocytes per high-power field (magnification, ×1,000). After the smear was taken, a urethral swab sample was collected for culture of *Neisseria gonorrhoeae*. Then, an ear-nose-throat cotton-tipped aluminum swab (Copan, Brescia, Italy) was inserted 3 to 4 cm into the urethra and subsequently placed in a tube with 1.8 ml of SP-4 mycoplasma broth medium (13). This specimen was sent to Copenhagen and used for detection of *C. trachomatis* and *M. genitalium* by PCR. Information regarding urethritis symptoms, i.e., discharge, dysuria, and itch, was extracted from patients' charts.

Eighty-two specimens were randomly selected among those found to be positive for *M. genitalium* by a conventional PCR assay detecting the 16S rRNA gene (12) and confirmed by an independent assay detecting the *mgpB* gene (14). A total of 164 samples were randomly selected among the 16S rRNA gene PCR-negative samples by selecting the two negative samples consecutively collected following a positive sample. Since the present study was mainly considered a methodological study, the random selection of specimens led to repeat sampling of some of the patients. After testing of the specimens was completed, the specimens were identified on the patient level. For all patients having multiple specimens examined, only the first specimen was included in the clinical part of the study. Thus, a total of 217 patients were eligible for the clinical study; of these, samples from 58 patients were positive by the LightCycler assay.

Sample preparation for urethral swab specimens. A 100-μl aliquot of the swab specimen in SP-4 was mixed with 300 μl of a 20% (wt/vol) Chelex 100 slurry (Bio-Rad, Richmond, CA) in TE buffer, and the mixture was shaken vigorously for 60 s and incubated at 95°C for 10 min. After centrifugation at 20,000 × *g* for 5 min, the supernatant was used for PCR. Sample preparation was performed before the specimens were frozen, and *M. genitalium* was detected by a standard rRNA gene PCR assay (12). Subsequently, the specimens were frozen and later used for the TaqMan assay (11) and LightCycler assay described in the present study.

Preparation of standard DNA. DNA was extracted from harvest of a 2× 150-cm^2 culture of *M. genitalium* G37 by the use of the genomic-tip system (QIAGEN Blood and Cell Culture DNA kits). DNA purification was performed according to the manufacturer's instructions. The purity and concentration of the genomic DNA were determined by optical density measurements at 260 and 280 nm. A genome size of *M. genitalium* of 580 kbp (7) was used to calculate the concentrations in copies per microliter. Dilutions of 10^5, 10^4, 10^3, 10^2, 10, 5, and 1 genome copies/μl of the purified DNA were used as standards for each LightCycler-PCR run. To prevent DNA from adhering to the walls of the plastic tubes, yeast RNA (10 μg/ml) was added to the dilution series as a "carrier" nucleic acid, and siliconized tubes were used.

LightCycler assay. Primers and probes were designed for amplification of a 190-bp product of the *gap* gene of *M. genitalium* (National Center for Biotechnology Information accession no. U39710 [http://www.ncbi.nlm.nih.gov]). The *gap* gene encodes the enzyme glyceraldehyde-3-phosphate dehydrogenase of the glycolysis pathway. The following primers and probes were used: forward primer, mg-gap-605f (5′-GTG CTC GTG CTG CAG CTG T-3′; nucleotides [nt] 605 to 623), reverse primer mg-gap-794r (5′-GCT TGA TTT ACT TGT TCA ACA GAT GGA C-3′; nt 767 to 794), a fluorescein (FL)-labeled probe, mg-gap-669FL (5′-TGT TGT TCC AGA AGC AAA TGG CAA ACT T-FL-3′; nt 669 to 696), and the LightCycler (LC) Red640 probe, mg-gap-700LC (5′-LCRed640-GGG ATG TCA CTC CGT GTT CCA GTG T-phosphate; nt 700 to 724). DNA Technology A/S, Aarhus, Denmark, synthesized the primers; TIB Molbiol, Berlin, Germany, synthesized the hybridization probes.

PCR reagents were mixed as follows: 0.5 μM each primer, 0.2 μM each probe, 5 mM $MgCl_2$, 2.0 μl of FastStart Master Hybridization Probes (Roche Diagnostics) (containing the hotstart *Taq* DNA polymerase, nucleotides [dGTP, dCTP, dATP, and dUTP instead of dTTP], and optimized buffer), and 2 μl of template DNA in a total volume of 20 μl. To prevent carryover, the mixture was incubated with 1.5 U of uracil-DNA-glycosylase (UNG) (Roche Diagnostics) for 10 min at room temperature before the PCR was initiated. UNG destroys PCR products containing dUTP. To avoid other DNA contamination, the PCR reagents were mixed in a PCR clean room, and the template samples were loaded with filter tips. The LightCycler instrument (Roche Diagnostics) was used for real-time quantitative PCR and the PCR program used was Hotstart at 95°C for 10 min (activates the *Taq* DNA polymerase and inactivates UNG); 45 cycles with denaturation at 95°C for 15 s, annealing at 55°C for 8 s, and extension at 72°C for 8 s. The temperature transition rate was 20°C/s. Fluorescence (640 nm) emitted by fluorescence resonance energy transfer from mg-gap-669FL to mg-gap-700LC was measured in channel F2/F1 when the probes annealed to the *gap* target DNA. After PCR, a melting program completed the analysis. The samples were heated to 95°C (20°C/s) without hold, cooled to 50°C (20°C/s) for 15 s, and slowly reheated to 95°C (0.1°C/s). At the end, the samples were cooled to 40°C (20°C/s) for 30 s.

Spiking of clinical samples. The LightCycler PCR assay was used to determine the PCR efficiency in the clinical samples. Twenty *M. genitalium* PCR-negative samples were chosen at random and spiked with 10^2 copies/μl of *M. genitalium* standard DNA. LightCycler PCR was performed on a dilution series of *M. genitalium* standard DNA and the spiked samples. Fluorescence data were exported directly from the LightCycler software 3.5 (Roche Diagnostics). Using SARA version 1.0 (http://www.gram.au.dk), the data for each curve were fitted with a four-parametric sigmoid model, using the following equation:

$$f = y_0 + \frac{a}{1 + e^{-\left(\frac{x - x_0}{b}\right)}} \quad (1)$$

using weighted least squares. In equation 1, f is the fluorescence (F2/F1) at cycle x, y_0 is the background fluorescence, a is the curve height, e is the natural logarithm base, x is the actual cycle number, x_0 is the second derivative maximum of the function, and b is the slope of the curve. The smaller the value of the slope b, the higher is the amplification efficiency (21). The slope generated from the spiked samples was compared to the slope of the standard, 10^2 copies/μl. The concentration of the spiked DNA was calculated from the standard curve and compared to the 10^2-copy/μl standard.

Sequencing. Part of the *gap* gene from the four M-strains and type strain G37 containing the amplicon was sequenced to verify the conservation of the primers and probes. Primers used for sequencing were placed upstream and downstream to mg-gap-605f and mg-gap-794r. The forward primer was mg-gap-532f (5′-GGA ACG ATG CTA ACA GTT CAT GC; nt 532 to 554) and the reverse primer was mg-primer-876r (5′-ACC ATA TTC AGA ACT TAC CAC ATC GC; nt 851 to 876). The PCR program used for amplification with this primer set was 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR was performed with a Perkin-Elmer GeneAmp PCR system 9600. The amplicon was sequenced in both directions with the mg-gap-532f and mg-primer-876r primers, respectively, using the ABI PRISM BigDye Primer Cycle Sequencing Ready Reaction kit (Applied Biosystems). Sequencing was performed with a Hitachi 3100 Genetic Analyzer (Applied Biosystems).

Sequencing was also performed on amplicons showing a different melting temperature than the standard DNA in the melting analysis. These amplicons were sequenced in both directions with mg-gap-605f and mg-gap-794r from the LightCycler assay. The sequencing was performed as described above.

Computer analysis. Analysis of the primer and probes was performed by use of the Genetics Computer Group (Madison, Wis.) program package (Wisconsin Package Version 10.1-unix) and BLAST database searches.

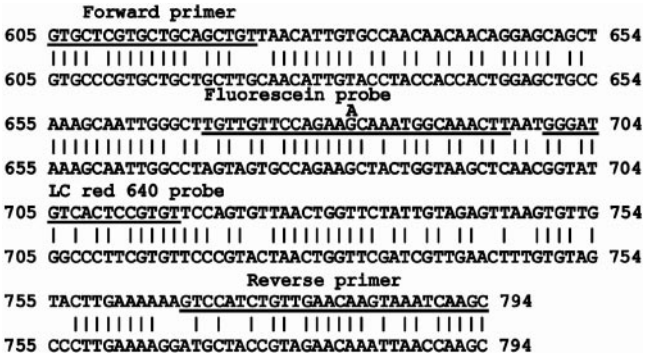

FIG. 1. Sequence of *gap* corresponding to the amplicon of the *M. genitalium* LightCycler assay. The *gap* sequence of *M. genitalium* (upper sequence) was compared to the homologous *gap* sequence of *M. pneumoniae* (lower sequence) by use of the GCG program Gap alignment (BioBase; www.biobase.dk). The selected primers and probes in the *M. genitalium gap* sequence are underlined. Primers were designed to differ from the *M. pneumoniae* sequences, especially at the 3′ ends. The probes were located on the same strand as the forward primer; to ensure good quantitative detection, they were placed closest to the reverse primer. Furthermore, 12 mismatches to *M. pneumoniae* were present in the probe region. In one *M. genitalium*-positive patient sample, a sequence analysis of the PCR product showed that guanine was replaced by adenine in position 682, as marked on the figure.

The fluorescence data were analyzed by LightCycler software, version 3.5. The fit point method was used (two fit points) to determine crossing points and concentrations.

Other PCR assays. All patients' specimens included in this study were also tested by a conventional 16S rRNA gene PCR assay as described previously by Jensen et al. (12).

The LightCycler PCR assay was compared to the recently developed real-time quantitative TaqMan PCR assay where *mgpB* encoding the adhesion protein MgPa is the target gene. The method is described by Jensen et al. (11).

Statistical methods. The logarithm of the DNA concentrations determined by the LightCycler and the TaqMan assays were compared by linear regression analysis with log concentration of the TaqMan assay as the dependent variable. The parameter estimates from the regression analysis were tested using a standard *t* statistic; however, to account for the pronounced heteroscedasticity, White-corrected standard errors were used (28).

For comparison of clinical data, Fisher's exact test was used. The Mann-Whitney test was used to calculate *P* values for the relation between median DNA load and urethritis.

RESULTS

Primer and probe design. The housekeeping gene *gap* of *M. genitalium* type strain G37 was chosen as the target for amplification because it is supposed to be relatively conserved among isolates and different from other species, including the *gap* homologue of *M. pneumoniae* (72.3% identity). Furthermore, there is only a single copy of the gene in the genome (7). Because of an identity of 98% of the 16S rRNA gene of *M. genitalium* and *M. pneumoniae*, the gap gene was chosen over the 16S rRNA gene, which was previously utilized for *M. genitalium* detection (12). Primers and probes were designed to be specific, which was determined by alignment with *gap* of *M. pneumoniae* (Fig. 1). The probes annealed to the same strand as the forward primer. Hence, the probes were placed nearest to the reverse primer to ensure optimal fluorescence detection in the annealing/extension phase. The sequence of the amplicon and the position of the primers and probes are shown in Fig. 1.

LightCycler PCR. A standard dilution series (10^5, 10^4, 10^3, 10^2, 10, 5, and 1 genome copies/μl) of the type strain G37 DNA was amplified in each LightCycler PCR run (Fig. 2, top). Fluorescence curves down to 1 genome copy/μl could be detected. Since 2 μl was added per reaction tube, the PCR was able to detect down to two copies of the genome. The standard curve is depicted in Fig. 2, bottom. The slope of the standard curve was −3.275, resulting in a calculated efficiency of 2.0 (100%), indicating that the number of PCR products was doubled in each cycle. The concentration of *M. genitalium* in patient samples was calculated from the standard curve.

Reproducibility. To determine the reproducibility of the LC-PCR, the intra- and interassay variations were measured with respect to crossing points and concentration. To determine the intra-assay variation, a maximum of four standard series were tested in one run. The coefficients of variation (CV) of the crossing points were in the range of 0.1% to 2.9%; the CV concentrations were low for the highest concentrations, ranging from 1.1% to 3.9%, and large in the last concentrations, ranging from 7.6% to 51.4%. Nine standard curves were evaluated for the determination of the interassay variation. All the standard curves were chosen consecutively from this project, and each run was performed on different days. The CV crossing point varied from 3.3% to 1.6%, and the CV concentration varied from 7.7% to 41.7%. All the different concentrations in the standard dilutions were detected in nine out of the nine runs except for the 1-copy/μl standard that was detected only five times out of the nine runs (55.5%) (Table 1). Since 5 copies/μl were detected in every run and 1 copy/μl was not, we decided to test the reproducibility of 2.5 and 1.25 copies/μl to approach the limit of detection for the assay. Both concentrations were detected in nine out of nine runs (Table 1); thus, the detection limit of the assay was <2.5 copies/reaction.

Specificity. The gap gene was sequenced from the M-strains and from G37 to examine the variability in the amplicon region. The sequencing of the *gap* gene corresponding to the PCR product showed that the sequences of the M-strains were identical to type strain G37. The four M-strains were also tested in the assay. In agreement with the results from sequencing, all the M-strains were detected, and the fluorescence curves were comparable to the 10^5 standard.

The assay proved to be specific for *M. genitalium*, since no cross-reactions were detected when DNA from the other mycoplasmas, *Chlamydia trachomatis*, and human DNA listed in Materials and Methods was tested.

Evaluation with patient samples. The LC *gap* assay was evaluated with 246 urethral specimens from men attending an STD clinic. Eighty-two specimens were *M. genitalium* positive by the 16S rRNA gene conventional PCR assay (12); the samples were from a subpopulation of the patients included in the study of Jensen et al. (11). The remaining specimens were randomly selected among those tested negative by the 16S rRNA gene assay (12). Of the positive patient specimens, 78 (95.1%) were found to be positive by the LightCycler assay and 4 (4.9%) were negative. The positive samples had a concentration of 1 to 2.5 × 10^4 copies/μl. For the negative patient specimens, 158 (96.3%) were found to be negative, but 6 (3.7%) were found to be positive, but with very low copy numbers (2 to 12 copies/μl). The LightCycler PCR was repeated with the six positive samples, and this time only one was

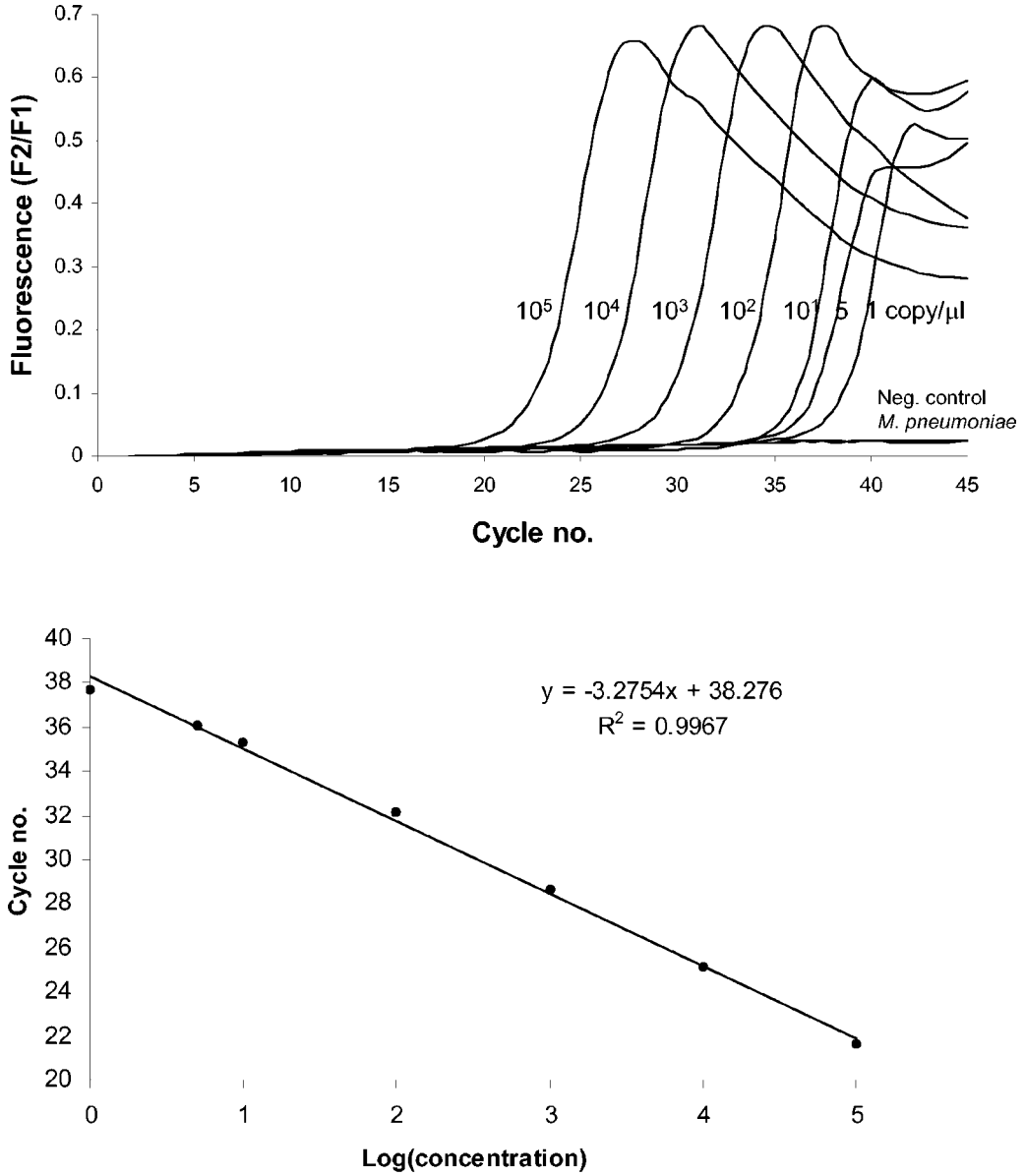

FIG. 2. (Top) Fluorescence curves of the standard dilution series. Fluorescence curves from 10^5 copies/μl down to 1 copy/μl are shown. Two negative controls are also included: one without DNA and one with *M. pneumoniae* DNA (10^5 copies/μl). F2 is the channel at which the fluorescence is measured at 640 nm. F1 is the channel at which the fluorescence is measured at 530 nm. (Bottom) Standard curve used to calculate the concentration of *M. genitalium* DNA in unknown samples. The PCR cycle number is plotted against the log concentration of the standard dilutions. The linear regression coefficient is close to 1, and the $10^{(-1/\text{slope})}$ value is ≈2.0, indicating a 100% efficiency of the PCR.

positive. Two of the six specimens, including the one repeatedly positive specimen, were positive by the TaqMan assay and should be considered true positives not detected in the 16S rRNA gene PCR.

Spiking assay. To investigate possible inhibition in the patient samples, a spike assay with 10^2 copies/μl of *M. genitalium* standard DNA was performed on 20 randomly selected *M. genitalium*-negative samples. The fluorescence data were fitted with a four-parametric sigmoid model. An example of a fitted curve (the 10^2-copy/μl standard curve) is shown in Fig. 3. As described by Tichopad et al. (21), a good *b* resolution was found if the coefficient of determination r^2 was >0.999. The resulting *b* values, r^2 values, and genome concentrations are shown in Table 2. The mean *b* value was 0.85 (standard deviation = 0.045, standard error of the mean = 0.010), and a *t* test showed that the *b* value of the spiked clinical samples was not significantly different from the *b* value of the standard curve ($b = 0.86$, $P = 0.148$). The mean concentration of the spiked clinical samples was 108.5 copies/μl (standard deviation = 22.45, standard error of the mean = 5.02, CV concentration = 20.7%) and did not differ significantly from the standard value of 115.3 copies/μl ($P = 0.188$). Thus, the PCR conditions seem to be comparable in clinical and standard samples.

TABLE 1. Interassay reproducibility of the LightCycler *M. genitalium* PCR assay

Genome copy no./μl	Observed no.	Mean CP[a]	CV[b] CP (%)	Mean concn	CV concn (%)
10^5	9/9	22.5	3.3	1.24×10^5	13.5
10^4	9/9	26.1	2.7	1.02×10^4	9.3
10^3	9/9	29.6	2.0	8.67×10^2	7.7
10^2	9/9	33.1	1.6	7.54×10^1	21.4
10^1	9/9	36.1	1.2	9.48	19.3
5	9/9	36.9	2.3	5.74	36.4
2.5	9/9	37.1	1.6	3.2	22.7
1.25	9/9	38.0	1.7	1.6	30.9
1	5/9	38.6	2.1	1.61	41.7

[a] CP, crossing point.
[b] CV, coefficient of variation.

TABLE 2. Spiking assay of 20 *M. genitalium*-negative urethral samples

Curve name	*b* (slope)	r^{2b}	Concn (copy/μl)
10^{2a}	0.86	0.9998	115.3
7012	0.83	0.9994	108.2
7014	0.86	0.9996	92.9
7134	0.78	0.9993	69.9
7136	0.85	0.9995	72.1
7173	0.85	0.9993	119.3
7178	0.81	0.9993	116.8
7227	0.90	0.9995	101.7
7229	0.90	0.9994	100.6
7277	0.83	0.9993	90.8
7279	0.76	0.9990	115.2
7292	0.81	0.9994	130.2
7294	0.85	0.9996	87.8
7303	0.95	0.9995	142.7
7326	0.82	0.9993	108.0
7345	0.83	0.9993	116.0
7350	0.81	0.9994	96.5
7387	0.90	0.9995	158.9
7389	0.88	0.9995	111.8
7560	0.81	0.9993	92.8
7571	0.88	0.9997	136.8

[a] Standard curve.
[b] r^2, coefficient of determination.

Comparison with the TaqMan assay. The 82 positive specimens were tested with a TaqMan assay (11), and the results were compared to the LightCycler assay. The assays were performed on the same pretreated specimen, which had been stored at −20°C, and were carried out independently in two laboratories. The TaqMan assay detected 81 out of the 82 positive specimens; 1 specimen was negative by both assays. The three samples that were negative by the LightCycler assay but positive by the TaqMan assay contained very low copy numbers (range, 0.03 to 2.65 copies/μl). When the *M. genitalium* DNA quantity as determined in the two assays was compared, the results correlated well (Fig. 4). Obviously, the spread of the observations was more pronounced for quantities of <10 copies/μl, in good agreement with the higher CV value for smaller quantities. By linear regression analysis, the slope was calculated to be 0.99, which was not significantly different from 1 when *t* statistics was applied ($t = -0.327$; $P = 0.744$). The linear correlation coefficient was determined ($R^2 = 0.894$), indicating that correlation between the data produced by the two PCR assays was rather good. The intercept was calculated to be −0.30, which was significantly different from 0 ($t = -2.96$; $P = 0.0041$). As a result of this, the trend line was not placed on top of the 45° dot-and-dash line (Fig. 4). Consequently, the average bacterial load determined by the LightCycler assay was 0.5 (intercept = $10^{-0.30} = 0.5$) times the concentration determined by the TaqMan assay.

Comparison of clinical signs and symptoms among *M. genitalium*-positive and -negative patients. Of the 246 urethral swab specimens tested by the LightCycler assay, 29 specimens were excluded due to repeated sample collection and 12 specimens were excluded because urethral smears were not done.

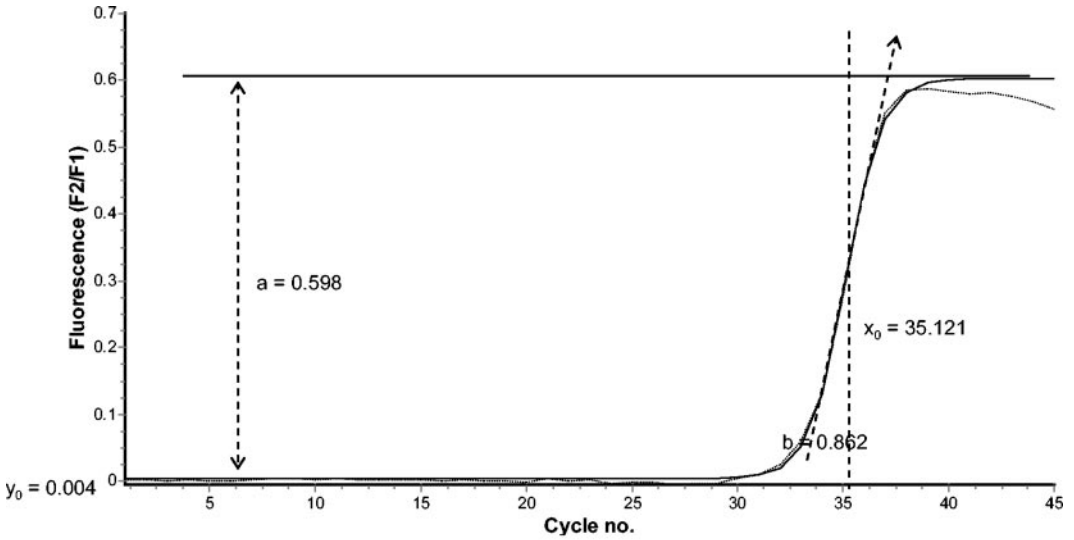

FIG. 3. The four-parametric sigmoid model (described by equation 1) used on fluorescence data from the spiking assay. In this example, the standard curve of 10^2 copies/μl is shown. The background fluorescence is designated y_0, *a* is the height of the curve, x_0 is the first derivative maximum of the function, and *b* is the slope of the curve.

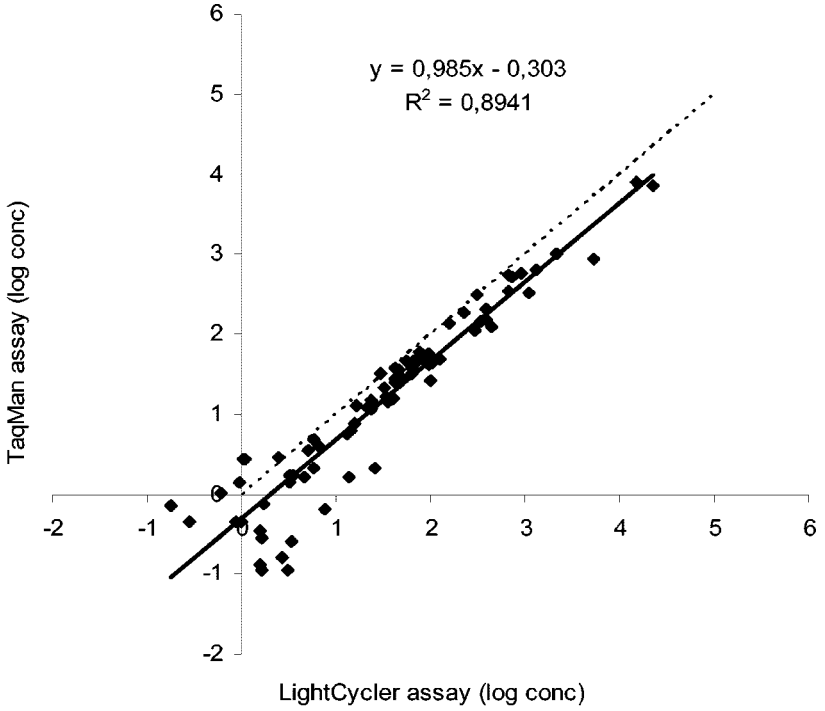

FIG. 4. The LightCycler assay was compared to the TaqMan assay (12). Seventy-eight patient samples were found positive and quantified by both PCR assays, and the results were plotted against each other. The linear regression line is shown as a solid line, whereas the expected 1:1 line is shown as a dotted line. Note the high linear correlation coefficient of 0.89 and the proximity to the 1:1 line. One specimen was negative by both assays; an additional three specimens were negative by the LightCycler assay but positive by the TaqMan assay (not shown).

Thus, a total of 205 specimens from the same number of men were included in analysis of the clinical data.

The patients were divided into two groups depending on whether the swab sample was positive (cases) or negative (controls) for *M. genitalium* by the LightCycler PCR. The results were compared with the clinical data: urethritis, self-reported discharge, dysuria, and itch (Table 3). Of the LightCycler-positive specimens ($n = 58$) 72% were from men with urethritis, which was a significantly higher proportion than the 35% among specimens from men without *M. genitalium* ($n = 147$; $P < 0.0001$) and 25% among the *C. trachomatis*-negative control specimens ($n = 122$; $P = 0.0005$).

Among the 58 cases, a significantly higher DNA load was found in specimens from men with urethritis (median, 61 copies/μl) than in specimens from those without (median, 2.9 copies/μl) ($P = 0.0005$; Mann-Whitney test).

Significantly more *M. genitalium*-positive men reported discharge (59%) than the controls (15%; $P < 0.0001$). This difference was even more pronounced when *C. trachomatis*-positive men in the control group were excluded (11%; $P = 0.0002$). Dysuria was reported by 62% of the *M. genitalium*-positive men compared to 28% of the controls ($P < 0.0001$) and 24% of the *C. trachomatis*-negative controls ($P < 0.0001$). However, the percentage of men with urethral itch was the same, whether or not they were positive for *M. genitalium*, and constituted 7% ($P = 1$).

C. trachomatis was found more often in the *M. genitalium*-negative men (17%) than in the *M. genitalium*-positive men (7%), although this difference did not reach statistical significance ($P = 0.075$). Among the *M. genitalium*-negative controls, 17% of patients were *C. trachomatis* positive and 84% of those had urethritis.

Among the 205 men included in the analysis of clinical data, 93 had urethritis, 42 (38%) were *M. genitalium* positive, and 24 (21%) were *C. trachomatis* positive. These proportions were both significantly higher than those found in men without urethritis, where 16 (14%) were *M. genitalium* positive ($P < 0.0001$) and 5 (4%) were *C. trachomatis* positive ($P < 0.0001$).

Melting curve analysis of clinical samples. A melting curve analysis was included to detect variability in the probe-binding region. There was no difference between the melting point (T_m) of the type strain G37 and the M-strains (68°C). Of the 78 samples found positive by LightCycler PCR, three samples had a decreased T_m of 64°C. The amplicons of these samples were sequenced and found to be different at position 682, where guanine was replaced by adenine (Fig. 1). All three samples derived from the same patient. Therefore, only one (1.7%) specimen out of the 58 LightCycler-positive men contained an amplicon with a mutation in the probe region. The LightCycler assay determined the concentration in the three samples to be 157, 677, and 306 copies/μl compared to 138, 543, and 305 copies/μl determined by the TaqMan assay.

DISCUSSION

We have developed a rapid, sensitive, and specific real-time PCR for the detection of *M. genitalium*. The LightCycler PCR is faster than conventional PCR. The PCR run and subsequent data analysis could be completed in approximately 50 min. The detection limit of the PCR was excellent, as the PCR could

TABLE 3. Comparison of LightCycler results with clinical data, i.e. urethritis, self-reported discharge, dysuria, and itch

Clinical symptom	*M. genitalium*-positive cases (%) (n = 58)	*M. genitalium*-negative controls (%) (n = 147)	P value[a]	*C. trachomatis*-negative controls (%) (n = 122)	P value[b]
Urethritis	42 (72)	51 (35)	<0.0001	30 (25)	<0.0001
Discharge	34 (59)	22 (15)	<0.0001	13 (11)	<0.0001
Dysuria	36 (62)	41 (28)	<0.0001	29 (24)	<0.0001
Itch	4 (7)	10 (7)	1	7 (6)	0.738

[a] P values compare results for *M. genitalium*-positive and -negative cases.
[b] P values are for results for *C. trachomatis*-negative samples.

detect down to two copies per reaction tube. However, due to the Poisson distribution of targets in dilute specimens, the realistic limit of detection was ≥5 copies per assay. The accuracy of this detection limit estimate was documented by the intra-assay reproducibility test, where nine replicates of 1 copy/μl were evaluated. Of these replicates, 33% were negative. If the DNA is assumed to be randomly distributed in the solution and to follow Poisson statistics, then the PCR is expected to miss $\exp(-2) \times 100\% = 13.5\%$ positives when an average of two DNA copies is added to the tube (corresponding to the concentration of 1 copy/μl). The difference between the expected and observed number of false negatives can be explained by pipetting errors (especially for low DNA concentrations), too few replicates, or a small overestimation of the DNA concentration in the *M. genitalium* genomic DNA standard solution. Other real-time PCR methods have been developed for the detection of *M. genitalium* in urine samples (6, 29). The limits of detection of the TaqMan-based assay by Yoshida et al. (29) and the LightCycler PCR assay by Dupin et al. (6) were similar to the sensitivity of our LightCycler PCR, with detection limits of 10 and 5 copies per reaction tube, respectively. However, since no external reference standard exists, only a direct comparison using a blinded panel of several DNA specimens could indicate true differences in the detection limits between assays. Furthermore, the more relevant performance estimate given by sensitivity and specificity can only be provided with genuine clinical specimens. To address this issue, we applied two independent real-time PCR assays targeting two different *M. genitalium* genes. To our knowledge, this is the first comparison between two real-time methods for the detection of *M. genitalium*. We found an excellent 1:1 relationship between the two PCR methods, as the slope of the regression line was very close to 1 (0.99), indicating that the quantity determined by the two assays was comparable. On average, the TaqMan assay estimated the level of *M. genitalium* DNA to be twofold higher than was estimated by the LightCycler assay. This was determined from the intercept of the regression line (Fig. 4). However, since this factor was constant over the detected range of *M. genitalium* DNA loads, it could probably be traced back to different standards used for determination of the standard curve and should not impede the detection of relative changes measured within each assay.

In validation of the reproducibility, the CV of the concentration was markedly increased for concentrations of $<10^2$ copies/μl (Table 1). This variation in the low-copy-number region was clearly demonstrated the results shown in Fig. 4, where the concentrations determined by the two PCR methods were plotted against each other. This increased variation for low copy numbers is probably due to the logarithmic scale and natural random variation.

In the clinical validation, the LightCycler assay failed to detect four specimens (4.9%) that were found positive by the 16S rRNA gene assay. Three of the four negative samples were found positive by the TaqMan assay, and the copy number proved to be very low (<3 copies/μl). Therefore, these differences can be explained by the Poisson statistics described above. If three or more replicates were tested, this difference would decrease. Obviously, the TaqMan assay has the advantage of being performed with a total volume of 50 μl, allowing for 5 μl of template DNA to be analyzed. However, this is more expensive in terms of reagents; if three replicates were to be analyzed, it would be more time consuming because fewer specimens could be included in each setup. Improved sample preparation protocols and the inclusion of an internal processing control in the LightCycler assay could increase the sensitivity but could also lead to a higher risk of sample-to-sample cross-contamination during more elaborate sample preparations. Six samples negative by the 16S rRNA gene assay were positive in very low copy numbers by the LightCycler assay. Two of these were also positive by the TaqMan assay, indicating that they were false negatives by the conventional assay. After repeated testing of the six samples, only one was found to be positive (0.6%). The Poisson distribution or, less likely, contamination may explain this finding.

The spiking assay showed that there was no measurable inhibition in the Chelex treated samples when compared to the QIAGEN-purified *M. genitalium* standard DNA. The average slope of the spiked curves was not significantly different from the standard curve, which means that the PCR efficiency is the same in the Chelex-treated samples and the QIAGEN-purified samples.

The sequences of the *gap* amplicons of the M-strains were identical to type strain G37. Fifty-seven of the 78 LightCycler-positive clinical samples had the same melting point, indicating an identical probe region. Only three samples obtained from the same patient differed in the melting analysis; sequencing revealed that only one base located in the middle of the first probe was exchanged. Thus, the expectation that the *gap* gene is relatively conserved in the amplicon region was verified. The bacterial load in the three samples determined by the LightCycler assay was in agreement with the concentrations determined by the TaqMan assay. Even if the average difference of 0.5 in the determination of the bacterial load between the two quantitative assays was considered, the single mutation in the probe region did not seem to affect the LightCycler quantification. No cross-reactions were found with any of the tested

organisms or to human DNA; more importantly, a good agreement was found between the two quantitative assays when applied to negative clinical specimens.

In the study by Yoshida et al. (29) the forward and reverse primers were 100% identical with *M. pneumoniae*. This is problematic if *M. pneumoniae* exists in the genital tracts, as has been previously described (9). It also restricts the PCR method to identify *M. genitalium* elsewhere, for instance in the respiratory tract where *M. genitalium* has also been isolated (2, 22).

As expected, there was a significant association between *M. genitalium* and urethritis in our study. A significant difference in the bacterial load between patients with and without urethritis was shown, but high DNA loads in patients without urethritis were also demonstrated, limiting the usefulness of quantification as a diagnostic predictor.

In conclusion, we have demonstrated that real-time quantitative PCR is a powerful tool to accurately determine the *M. genitalium* load in clinical samples. A good quantitative method is needed in clinical studies, as well as in the laboratory, because culture is too difficult to be an alternative.

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H. F. Svenstrup initiated the study; designed, optimized and evaluated the LightCycler assay; performed the assay and data management; and wrote the first draft of the manuscript. J. S. Jensen provided the clinical specimens with known *M. genitalium* PCR status and TaqMan *M. genitalium* quantitative data and provided major contributions to the design of the study and analysis of the data. E. Björnelius and P. Lidbrink collected the clinical specimens. G. Christiansen and S. Birkelund supervised the study, provided research facilities, and commented on the manuscript.

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